STANDARD OPERATING PROCEDURE
NHP GENOMICS CORE LABORATORY

Subject: cDNA synthesis for Single cell using the Clontech SmartER chemistry	**SOP Number:** 69.1 **Version:** 0
Effective Date: Jan 22, 2016	**Revised Date:** -

cDNA synthesis for Single cell using the Clontech SmartER V4 chemistry

Rationale:

Single cells separated by flow-cytometry is disrupted in Qiagen RLT buffer and the RNA stabilized for later purification and cDNA synthesis using the Clontech SmartER chemistry.

Materials:

Materials	Company	Cat. No.
RNAClean XP Beads	Beckman Coulter	A63987
96 Well magnetic stand	Ambion	AM10027
100% Ethanol	Fisher Scientific	BP2818500
SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing	Takara Bio	634891

A. Single Cell in RLT Bead-Cleanup

Notes:

- Before beginning, equilibrate RNAClean XP Beads at room temperature for at least 45 minutes.
- Dedicated set of pipettes, plates and bench space should be maintained for pre-amplification and post-amplification areas.
- The following steps should be done in a pre-amplification area of the lab.
- Clean all work surfaces and pipettes with RNAseZap and DNA-Off.
- Always prepare 80% ethanol fresh the day of the experiment, using nuclease-free water.
- The following methods are performed at room temperature.
- Please use 12 channel pipettes for bead clean-ups, always use filtered RNAse-free tips.

Methods:

1. Thaw plate containing single cells on ice.
2. Wipe down the plate carefully with lint free tissue to get rid of any condensate.
3. Vortex and centrifuge the plate to ensure all liquid is at the bottom of the wells. Gently peel off the aluminum foil.
4. Thoroughly vortex RNAClean XP beads to mix, invert the bottle at least 8-10 times.
5. Add 2.2 volume of the RNAClean XP beads to each well and mix by pipetting up and down 10 times (22 ul of beads to 10 ul of RLT).
6. Seal the plate with adhesive seal and centrifuge the plate.
7. Incubate sealed plate for 10 minutes on the benchtop.
8. While the plate is incubating, pull out 10X lysis buffer, RNAse inhibitor, 5X Ultra Low first strand buffer, 3' SMART-Seq CDS Primer II A (12 um), SMART-Seq v4 Oligonucleotide (48 um) and place them on ice to thaw.
9. Place the plate on the magnetic stand for 5 minutes, until the supernatant is clear and the beads are visibly bound to the magnet.
10. Remove the supernatant from each well, being careful not to aspirate the beads (remove 25 ul of supernatant).
11. Wash the beads by adding 150 ul of 80% ethanol to each well while the plate is on the magnet. Wait 30 seconds and then aspirate and discard the ethanol from the well.
12. Repeat the ethanol wash twice, so a total of 2 times.
13. Ensure that all ethanol is removed by using a P20 pipette.
14. Leaving the plate on the magnet, allow the beads to dry for 3-5 minutes, until the bead pellet is matte and a crack first appears.
15. Ensure the beads don't over dry, this is CRITICAL. Over dried beads will result in poor amplification and loss of yield.
16. While the beads are drying, prepare the Reaction Buffer and re-suspension Mix for bead re-suspension:

Reaction Buffer (RB)	
Reagent	**Volume per 18 reactions (ul)**
10X Lysis Buffer	1
RNase Inhibitor	19

Re-suspension buffer (RSB)	
Reagent	**Volume 1X (ul)**
Reaction Buffer	1
Nuclease-Free Water	10.5
3' Smart-seq CDS Primer II (12 uM)	1

17. Once the bead pellet has dried, remove the plate from the magnet and add 12.5 ul of RSB to each well, being careful to not disturb the beads with the pipette tip.
18. Gently vortex and centrifuge the samples until the beads are fully re-suspended.
19. Incubate the re-suspended beads at room temperature for two minute.
20. Incubate the plate at 72°C in a preheated, hot-lid thermal cycler for 3 minutes to anneal the 3' SMART-Seq CDS primer.
21. Immediately after the 3 minutes incubation at 72°C, place the samples on ice for 2 minutes. Proceed immediately to first strand cDNA synthesis.

B. First-strand cDNA Synthesis

Notes:

- Vortex and spin each reagent (except the enzyme) before use.
- Add enzymes just before use. Gently mix the tubes without vortexing.
- Ensure that the plate is kept on ice throughout protocol.

Methods:

1. Prepare the First Strand Mix, in the order below:

First Strand mix (FSM)	
Reagent	**Volume 1X (ul)**
5X Ultra Low First Strand Buffer	4
SMART-Seq V4 Oligonucleotide (48 um)	1
RNase inhibitor	0.5
SMARTScribe Reverse Transcriptase	2

2. Vortex gently to mix reagents and spin the tube.
3. Add 7.5 ul of FSM to each sample using a repeater pipette.

4. Seal the plate, gently vortex, and centrifuge to ensure that all liquid is at the bottom of each well.
5. Place the sealed plate on a thermocycler and run the following program:

Step	Cycle	Temperature (°C)	Time (minutes)
1	1	42	90:00
2	1	70	10:00
3	1	4	Hold

6. Immediately proceed to Second-strand Synthesis.

C. **Second-strand cDNA Synthesis**

Materials: SeqAmp DNA Polymerase (638509), 2X SeqAmp PCR Buffer (638509), PCR Primer IIA (12 uM), Nuclease-free Water

Notes:

- Vortex and spin each reagent (except the enzyme) before use.
- Add enzyme just before use. Gently mix the tube without vortexing.
- Ensure that plate is kept on ice throughout protocol.

Methods:

1. Thaw all reagents needed for second-strand synthesis on ice.
2. Prepare the PCR Mix, adding reagents in the order shown:

PCR Mix	
Reagent	**Volume 1X (ul)**
2X SeqAmp PCR Buffer	25
PCR Primer IIA (12uM)	1
SeqAmp DNA Polymerase	1
Nuclease-Free Water	3

3. Vortex gently to mix reagents and spin the tube.
4. Add 30ul of PCR Mix to each sample, using a repeater pipette.
5. Carefully seal the plate. Gently vortex and centrifuge the plate to mix.
6. Place the sealed plate on a thermo cycler and run the following program:

Step	Cycle	Temperature (°C)	Time (minutes)
1	1	95	1:00
2	18	98	0:10
		65	0:30
		68	3:00
3	1	72	10:00
4	1	4	Hold

Stopping Point: Samples may be stored at 4°C overnight.

D. Purification of cDNA using AMPure XP Beads

Materials: Agencourt Ampure XP Beads (A63881), 10X Lysis Buffer (635013), 80% Ethanol, Elution Buffer, magnetic stand (AM10027)

Notes:

- Before beginning, set out Ampure XP Beads at room temperature for at least 45 minutes. Ensure they are fully mixed before use.
- The following steps should be done in a **post-amplification area** of the lab.
- Clean all work surfaces and pipettes with DNA-Off before starting the clean-up.
- Prepare ethanol fresh the day of the experiment, using nuclease-free water.

Methods:

1. Make sure the Ampure XP beads are at room temperature and well mixed. Then add 50 ul of beads to each sample.
2. Mix well by pipetting up and down at least 10 times.
3. Incubate the plate at room temperature for 8 minutes.
4. Place the plate on the magnetic stand for 5 minutes, until the liquid is clear and the beads have visibly bound to the magnet.
5. Leaving the plate on the magnetic stand, remove the supernatant and discard (~98 ul). Be careful not to remove any beads.
6. Add 200 ul of 80% ethanol to each sample. Wait for 30 seconds and then remove the ethanol.
7. Repeat the ethanol wash twice, so a total of 2 times.
8. Ensure that all ethanol is removed from the wells by using a P20 pipette.
9. Allow the bead pellet to dry for 5-8 minutes, until the bead is matte and a crack first appears.
10. Once the beads are dry, remove the plate from the magnet and pipette 17ul of Clontech elution buffer over the beads, being careful not to disturb the bead with the pipette tip.
11. Thoroughly resuspend the beads by vortexing and centrifuging the plate.
12. Incubate the plate at room temperature for 2 minutes.
13. Place the plate on the magnet for 2 minutes, until the liquid is clear.
14. Transfer the supernatant of purified cDNA (~15 ul) to a new plate.
15. Proceed to Quality and quantity assessment, step 16, 17.
16. Run 1 ul of cDNA on a DNA High sensitivity chip on the Agilent Bioanalyzer. The good amplification trace is shown in figure 1.
17. Use 2 ul of the cDNA and quantitate on Qubit using the dsDNA High sensitivity chip.
18. Store the remainder of the cDNA at -80°C.

19. Follow the Illumina NexteraXT user guide to prepare barcoded libraries for sequencing.

Good quality

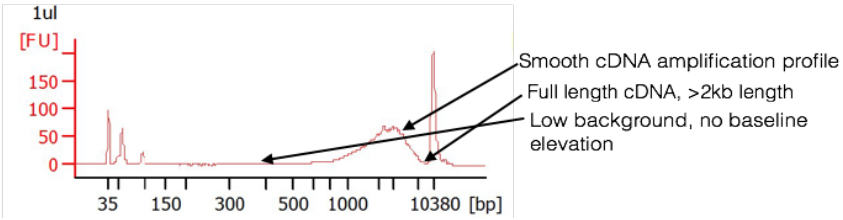

Figure 1: cDNA trace from DNA high sensitivity chip.